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Rat liver membranes contain a 120 kDa glycoprotein which serves as a substrate for the tyrosine kinases of the receptors for insulin and epidermal growth factor

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The receptors for insulin and epidermal growth factor possess tyrosine-specific protein kinase activity which may play a role in mediating the biological actions of these two peptides. We have identified a 120 kDa glycoprotein (pp120) in rat liver plasma membranes which can be phosphorylated by the insulin receptor in a cell-free system and in intact cultured hepatoma cells. In the present report, we have demonstrated in a cell-free system that solubilized epidermal growth factor receptors can phosphorylate tyrosine residues in pp120.

Insulin receptor; Epidermal growth factor receptor; Tyrosine kinase; Protein phosphorylation

1. INTRODUCTION

Receptors for insulin and several peptide growth factors possess tyrosine-specific protein kinase activity [1–5]. Recent evidence strongly supports the hypothesis that the receptor-associated tyrosine kinase activity plays a key role in mediating the biological actions of these peptides [6,7]. Nevertheless, the biochemical mechanisms by which this transmembrane signalling is accomplished have not been elucidated in detail. To address this question, several laboratories have attempted to identify substrates which are phosphorylated by these tyrosine kinases [8–13]. Previously, we have identified a 120 kDa glycoprotein (pp120) in rat liver plasma membranes which is phosphorylated by the insulin receptor in a cell-free system [8,9]. More recently, we have demonstrated that insulin stimulates phosphorylation of tyrosine residues in pp120 in intact hepatoma cells [10]. Here, we have demonstrated that epidermal growth factor (EGF) stimulates phosphorylation of pp120 by the rat liver EGF receptor.

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2. MATERIALS AND METHODS

2.1. *Preparation of detergent extracts of rat liver microsomes*

Microsomes were prepared from livers of 150–250 g male Sprague-Dawley rats as described in [8]. The microsomes (protein concentration, approx. 15 mg/ml) were solubilized for 30 min at 4°C in 50 mM Hepes (pH 7.8) containing 1% Triton X-100, and the extract was partially purified by chromatography over wheat germ agglutinin-agarose (Vector Laboratories, Burlingame, CA) [8].

2.2. *Protein phosphorylation*

The eluates from the wheat germ agglutinin-agarose were employed immediately for studies of

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protein phosphorylation. Although it was possible to study insulin-stimulated phosphorylation after freeze-thawing the samples, we found that EGF-stimulated phosphorylation could only be demonstrated when the extracts were not frozen. Aliquots (0.1 ml) of eluate (approx. 0.5 mg protein/ml) were combined with aliquots (0.15 ml) of Hepes (50 mM, pH 7.8) containing Triton X-100 (0.1%), plus 0.1 ml of the same buffer supplemented with bovine serum albumin (0.1 mg/ml) plus sufficient quantities of insulin or EGF to yield final peptide concentrations of $0–10^{-6}$ M. After incubation for 30 min at room temperature, phosphorylation was initiated by addition of 0.1 ml of a reaction mixture containing [γ-^{32}P]ATP (1 mCi/ml, 3000 Ci/mmol), ATP (1.9 mM), CTP (25 mM), and $MnCl_2$ (100 mM). An aliquot (0.1 ml) of the phosphorylation reaction was terminated by addition of 0.06 ml 'stopping solution' (16 mM EDTA, 320 mM NaF, 32 mM Na pyrophosphate, 3.2 mM Na vanadate, 50 mM Hepes (pH 7.8), 0.1% Triton X-100). For immunoprecipitation studies, 0.004 ml of either normal rabbit serum or R2-6 rabbit anti-pp120 antiserum [8] was added to 0.16 ml of this stopped reaction mixture. The immunoprecipitates were analyzed by SDS-polyacrylamide gel electrophoresis followed by autoradiography. In some studies (fig.1B), [^{32}P]phosphoproteins were analyzed directly without immunoprecipitation. In these studies, an aliquot (0.09 ml) of the phosphorylation reaction mixture (without addition of stopping solution) was added to 0.03 ml of 5 × Laemmli sample buffer containing 0.1 M dithiothreitol [14]. Thereafter, the solution was boiled and clarified by centrifugation; aliquots (0.055 ml) were analyzed by SDS-polyacrylamide gel electrophoresis followed by autoradiography.

2.3. *Phosphoamino acid analysis*

After pp120 was located by autoradiography of the wet gels, the ^{32}P-labeled bands were excised. After acid hydrolysis of the protein, phosphoamino acids were prepared for analysis and identified by high-voltage electrophoresis at pH 3.5 followed by autoradiography [8,15].

3. RESULTS AND DISCUSSION

As we have shown previously [8,9], insulin

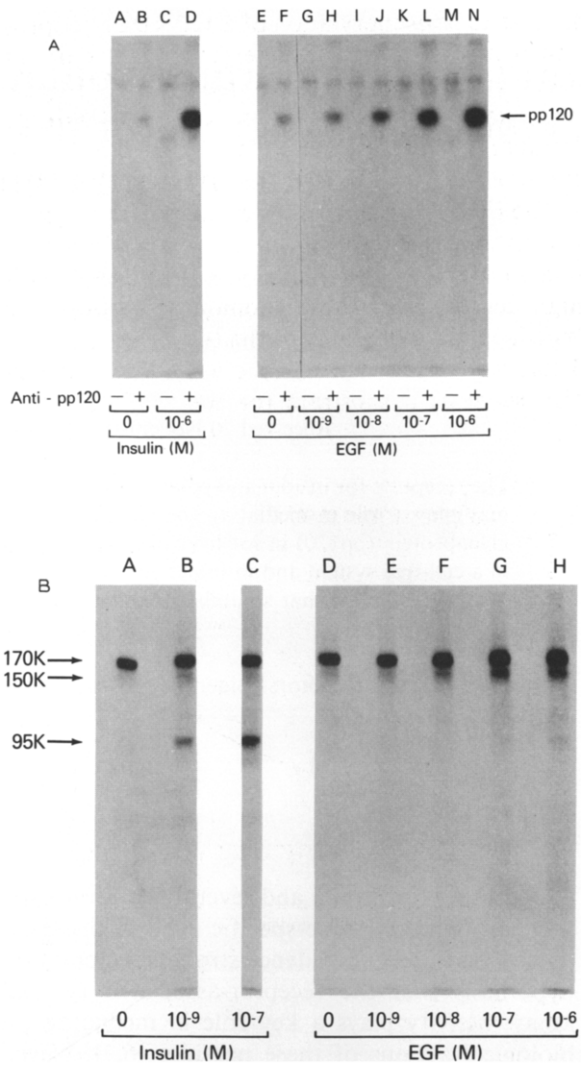

Fig.1. Stimulation of protein phosphorylation by insulin and epidermal growth factor. The eluate from the wheat germ agglutinin-agarose column was incubated with [γ-^{32}P]ATP in the presence or absence of insulin or EGF as indicated. To study receptor autophosphorylation (B), samples were mixed with 5 × Laemmli sample buffer and analyzed by SDS-polyacrylamide gel electrophoresis followed by autoradiography (4 h exposure). To study phosphorylation of pp120 (A), the reactions were terminated by stopping buffer (see section 2) and subjected to immunoprecipitation with either normal rabbit serum (lanes A,C,E,G,I,K,M) or anti-pp120 antiserum (lanes B,D,F,H,J,L,N). The immunoprecipitates were analyzed by SDS-polyacrylamide gel electrophoresis followed by autoradiography (16 h exposure).

stimulates phosphorylation of a 120 kDa glycoprotein which is present in detergent extracts of rat liver membranes (fig.1A, lanes A–D). This phosphorylation, which is specific for tyrosine residues on pp120, appears to be catalyzed by the tyrosine kinase activity of the insulin receptor [8].

The EGF receptor resembles the insulin receptor in that both receptors possess tyrosine-specific protein kinase activities [1,2]. Moreover, there is a high degree of structural homology between the tyrosine kinase domains of the two receptors [16]. Thus, we wished to determine whether pp120 can also serve as a substrate for the EGF receptor-associated tyrosine kinase. Rat liver membranes were solubilized in Triton X-100 and the glycoprotein fraction was purified by affinity chromatography over wheat germ agglutinin-agarose. The eluate was incubated in the presence of [γ-^{32}P]ATP plus varying concentrations of EGF. Subsequently, pp120 was immunoprecipitated by addition of an antiserum (R2-6) produced by immunization of rabbits with partially purified pp120 [8]. Under these conditions, EGF (10^{-6} M) caused an approx. 5-fold increase in ^{32}P incorporation into pp120 (fig.1A, lanes G–N).

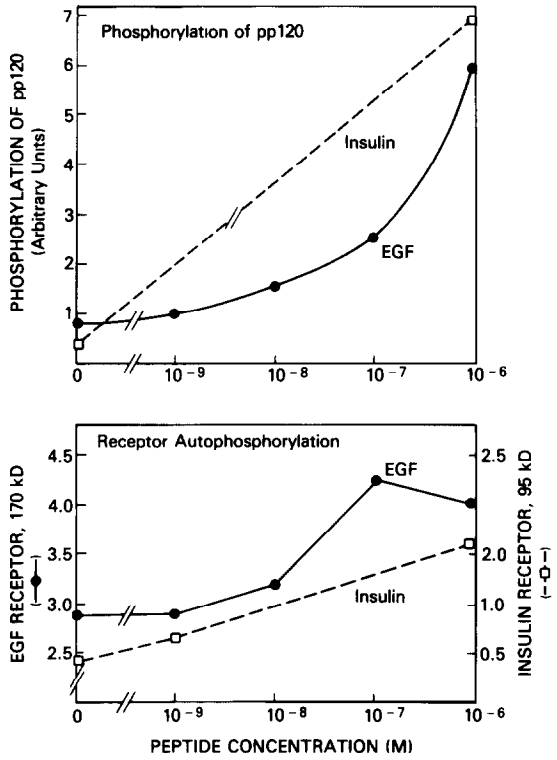

Fig.2. Dose-response relationship for epidermal growth factor-stimulated phosphorylation. The ^{32}P associated with pp120 (fig.1A), the 95 kDa subunit of the insulin receptor (fig.1B), and the 170 kDa EGF receptor (fig.1B) was quantitated using a scanning densitometer. EGF-stimulated phosphorylation is depicted by the solid lines. For purposes of comparison, the effects of a maximally effective concentration (10^{-6} M) of insulin as well as 10^{-9} M insulin (lower panel only) are depicted by the dashed lines. The detailed dose-response relationships for insulin have been published [8].

In the same experiment, we demonstrated that insulin (fig.1B, lanes A–C) and EGF (fig.1B, lanes D–H) stimulated autophosphorylation of their respective receptors. The dose-response curves for phosphorylation in the same experiment are presented in fig.2. As we have shown previously with insulin [8], the dose-response curve for stimulation of phosphorylation of pp120 by EGF closely parallels the dose-response for EGF receptor autophosphorylation. In four separate experiments, maximal effects of insulin and EGF to stimulate phosphorylation of pp120 were observed at concentrations of 10^{-7}–10^{-6} M (not shown). As was observed previously with insulin-stimulated phosphorylation, EGF stimulates phosphorylation of pp120 primarily on tyrosine residues (fig.3). Studies are currently underway to determine whether the two receptors phosphorylate the same tyrosine residues in pp120.

It is of obvious importance to define the physiological role for pp120 in the mechanism of action of peptide growth factors. Although this is not possible at the present time, several clues suggest some speculative possibilities. Because we have demonstrated that pp120 is a substrate for the EGF receptor as well as the insulin receptor, it is tempting to propose that pp120 may serve a function which is common to the actions of both insulin and EGF. For example, pp120 may play a role in mediating the effects of insulin and EGF to promote liver cell growth, as has been observed in hepatic regeneration after partial hepatectomy. The inference that pp120, which is located in the plasma membrane, plays a role in the regulation of cell growth is also consistent with recent studies of the requirements for cellular transformation by oncogenic viruses. That work has suggested that membrane proteins may be the most important

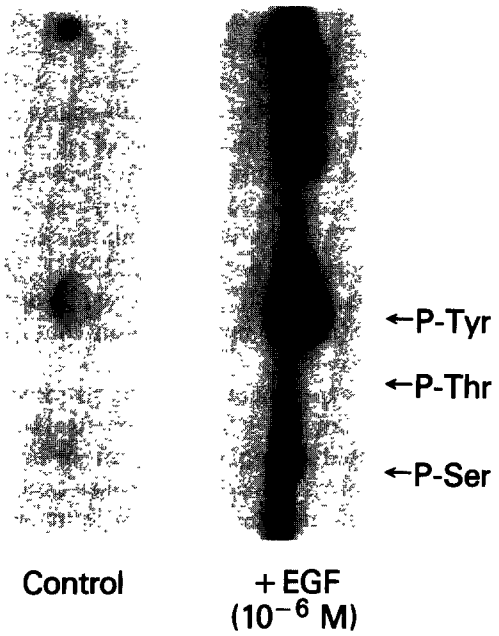

Fig.3. Phosphoamino acid analysis. Phosphorylation of pp120 was studied as usual with the exception that the [γ-^{32}P]ATP concentration in the reaction mixture was increased to 5.6 mCi/ml (spec. act. 3000 Ci/ml), and unlabeled ATP was omitted. The bands corresponding to pp120 were located using autoradiography and excised from the wet gel. Thereafter, phosphoamino acids were analyzed by high-voltage electrophoresis at pH 3.5 followed by autoradiography (see section 2).

targets for phosphorylation by viral tyrosine kinases [17].

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